

OPSONIZATION OF SALMONELLA TYPHI MURIUM
BY SPECIFIC ANTIBODY FRAGMENT I

INAUGURAL-DISSERTATION

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HANS STALDER

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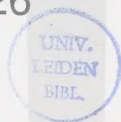
Department of Microbiology, College of Medicine
University of Florida, Gainesville, Florida

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INTRODUCTION

The first recognition of the capacity of certain cells to remove particles from the body fluids, be it blood or lymph, dates back almost 100 years. As Hoffmann and von Recklinghausen observed in 1867, cinnabar (HgS), when injected into the lymph sac of the frog, is fixed in liver, spleen and kidney. The cells which had picked up the cinnabar also were found to contain clumps of pigment and red cells (1). Additional experiments indicated (2, 3, 4) that this phenomenon of fixation of particles from the blood and lymph circulation was not limited to cold blooded animals but also occurred in mammals, such as rabbits and guinea pigs, and encompassed besides liver, spleen and kidneys, the blood leukocytes, bone marrow and lymph nodes. Shortly thereafter, it was recognized that microorganisms were fixed in a similar fashion as were inert particles (5, 6). On the basis of these and additional observations Metchnikoff developed the concept of cellular immunity with the phagocytes playing a crucial role in resistance to microbial invasion (6, 7). The classic description of the cellular involvement in inflammation by Cohnheim (8) was strong support for Metchnikoff's ideas (9) who distinguished on a morphologic basis between microphages (polymorphonuclear leukocytes) and macrophages (monocytes and histiocytes). Thus, cells of greatly differing morphology and distributed throughout the body exhibited the common property of phagocytosis, and were somehow essential for resistance to infection.

However, it was soon recognized by Bordet (10, 11) and by Denys and Lecleff (12) that serum was required to obtain phagocytosis, and more specifically, that there were two factors involved. The first was immunologically specific and heat stable, and the second was nonspecific and heat labile. Wright and Douglas (13) showed later that heat inactivation removed most of the phagocytosis promoting activity of serum, and they concluded that they had "proof that the blood fluids modify the bacteria in manner which renders them a ready prey to the phagocyte. We may speak for this as an opsonic effect (opsono = I cater for, I prepare victuals for) and we may employ opsonins to designate the elements in the blood which produce this effect".

These and other findings strongly suggest that phagocytosis, i.e. the actual ingestion of the particle, must be preceded by physical approachment of particle and cell resulting in contact and adhesion. It is likely that specific and nonspecific serum components are contributory to the latter. Phagocytosis then can be considered as a three component system namely:

1. The phagocytes, either from blood or tissues.
2. The surrounding fluid, either plasma or extravascular fluid, and
3. The physical and chemical attributes of the particle, usually a foreign body.

The experiments to be reported below were concerned only with an analysis of one of the serum factors, namely the specific antibody to a surface component of the particle used, *Salmonella typhi* murium and *Salmonella paratyphi* B. It seems sufficiently proven that immune bodies are the most potent

opsonins (10, 11, 12, 13, 14, 15) and that the rate of phagocytosis by the reticulo-endothelial system is within certain limits, proportioned to the amount of specific antibody present in the serum (16, 17). In addition, nonspecific γ -globulin has been shown to be an opsonin, at least for the clearance of inert particles (18). There seems little doubt that the thermolabile component of serum described by Wright and Douglas (13) refers to the complement system (19, 20, 21, 22). Complement (C') consists of at least 6 components some of which are heat labile, thus inactivating the entire system (for details see ref. 22). In spite of some reports to the contrary (20) serum of newborn piglets, which contains no detectable antibody, has an opsonizing effect on the rough strain of *Escherichia coli*. This effect is attributed to complement present in piglet serum (23). These contradictory findings indicate the difficulty in attempting to separate unequivocally the opsonizing effect of antibody and complement. Complement can either be involved as a participant in an antigen-antibody reaction or directly due to the anti-complementary action of the antigen on the surface of the particle. In addition to complement, there are other thermolabile components of serum with opsonizing effect (24). For all these factors Tullis and Surgenor (25) proposed the term "phagocytosis promoting factors", which in reality is admission of ignorance. Blockade of the reticuloendothelial system has been attributed to the depletion of such heat labile plasma components (19) rather than to the saturation of the phagocytes with particles (26).

Recent progress in the elucidation of the structure and function of antibodies especially of 7S γ -globulins provided new tools for the study of opsonization. Thus, Porter (27, 28) reported successful splitting of γ -globulin into 3 fragments, two of which, fragments I and II, contain the antibody sites and activity, whereas the third, fragment III, is devoid of specific sites but is held responsible for the combination of antibody with complement (29, 30, 31). In view of the considerable ignorance in regard to the mechanism of opsonization a comparative study was undertaken of the opsonizing effect of γ -globulin, fragment I and fragment III prepared from rabbit anti-Salmonella antiserum. It was essentially shown that fragment I indeed possesses some specific activity in influencing clearance of *Salmonella typhi* murium from the circulation of mice.

MATERIALS AND METHODS

(for general reference to techniques see 32)

Salmonellae - *Salmonella typhi* murium was obtained from Dr. Morris Tager, Emory University, Atlanta, Georgia, and *Salmonella paratyphi* B⁷⁷⁷ was a strain supplied by Dr. O.E. Landman, Fort Detrick, Maryland. The two strains share the somatic antigen but differ in their flagellar antigen (33). The antibody against the former is responsible for opsonization (20). The cultures were maintained on blood agar plates. For the experiments the organisms were grown in 40 ml of brain heart infusion broth either for 8 hours stationary or for 5 hours on the shaker.

Antisera - New Zealand white rabbits were immunized by repeated intravenous injections of washed bacillary suspension three times a week for three weeks. This course of injections was repeated twice. After a longer rest period the animals received another sequence of these injections and were bled from the ear vein five days after the last injection. The sera were collected and stored in the frozen state. The agglutination titer, when tested against *Salmonella typhi* murium was 1:256 for the anti-*Salmonella typhi* murium serum and 1:2048 for the anti-*Salmonella paratyphi* B serum.

Preparation of γ -globulin - Globulins were precipitated from serum in 0.37 saturated ammonium sulfate. The precipitate was washed twice in ammonium sulfate and redissolved in saline. The protein was dialyzed against 0.0175 M sodium phosphate buffer at pH 6.3 and chromatographed on a diethylaminoethyl (DEAE) cellulose column in the same buffer. Protein concentrations were determined in the Beckman DU Spectrophotometer at 280 m μ . The sedimentation coefficient (20°C, H₂O) of the chromatographed γ -globulin was determined in the analytical centrifuge (Spinco Beckmann, Model E) at 56,000 rpm and was found to be 6.8S (32).

Fragment I - The γ -globulin was split with soluble papain in a weight ratio of 1:100 at pH 8.0 using 0.1 M cysteine in 0.02 M versene as reducing agent. The mixture was incubated at 36°C for 12 hours and was then dialyzed against saline. For further purification of fragment I the papain digest was chromatographed on a carboxymethyl (CM) cellulose column in 0.02 M acetate buffer at pH 5.5. The solution of the fragment I proved to be free of detectable amounts of either γ -globulin or fragment III as evidenced by ultracentrifugation and agar gel precipitation analysis. As can be seen in Figures 1a and 1b, fragment I shows sharp lines of precipitation with an anti-rabbit γ -globulin antiserum, lines which show no sign of cross reaction with the line of precipitation formed by fragment III with the same antiserum. Ultracentrifugal analysis revealed one peak with a sedimentation coefficient of 3.5S. The diffusion coefficient was determined using a speed of 9341 rpm and was calculated to be $D = 7.0 \times 10^{-7}$ cm²/sec. From these data the molecular weight of the fragment of 44,000 was calculated.

Animals - For opsonization tests female Swiss white mice of the ICR strain were purchased from a commercial breeder (Springer, Dublin, Virginia). The animals weighed between 20 and 25 g and were fed pellets and water ad libitum.

Experimental procedure - The culture of *Salmonella typhi* murium, grown in the manner described above, was centrifuged in an international centrifuge at 1500 rpm for 10 minutes. The bacteria were resuspended in 10 ml of saline. Serial dilutions were plated on Difco nutrient agar for viable count. The counts ranged between 3.7 and 8.1×10^8 organisms per ml. Of this suspension a 1:1000 dilution was prepared in saline, of which 0.2 ml was injected intravenously per mouse. The mice, then, were injected with 0.8 to 1.6×10^5 organisms. In every experiment groups of mice were injected into one of the tail veins with 0.1 ml of γ -globulin, fragment I or saline respectively. Two to three hours later the mice were infected in the above described manner. Quantitative determinations of bacteria

in the circulating blood were done immediately, 1, and 24 hours after infection. The chest of the mouse which was deeply anesthetized with chloroform was opened aseptically, and 0.5 ml of blood was withdrawn from the heart using a plastic 1 ml syringe. Serial dilutions of blood were plated on nutrient agar for quantitative viable counts.

One mouse of each group was killed immediately after infection to obtain a value of the initial blood content of bacteria. Experiments showed that these values were fairly constant and that one mouse was sufficiently representative for a group. Table I shows that the values derived from 5 normal and 5 passively immunized mice are almost identical, namely $\log_{10} 4.75$, $S = 0.15$ and 4.85 , $S = 0.05$ respectively. Most of the other mice were killed after 1 hour, however in one group after 24 hours.

CALCULATIONS AND PRESENTATION OF RESULTS

For calculation of means of number of bacteria per ml blood and the standard deviation $\log_{10}$ were used. Frequency diagrams were prepared by plotting the number of organisms per ml blood in increments of 10 on the abscissa and the percentage of mice containing the corresponding number of bacteria per ml blood on the ordinate.

RESULTS

The effect of γ -globulin and fragment I prepared from anti-Salmonella typhi murium antiserum - The results are presented in Table II and Figure 2. Since the γ -globulin appears to have no effect on the number of organisms found immediately after infection (see Table I), only control animals were sacrificed at that time. The findings after one hour are quite striking. While the organisms persisted almost completely in the blood of control animals, no bacteria were found in those immunized with γ -globulin. The mice injected with fragment I showed an average number of organisms persisting in the circulation intermediary between the controls and the mice protected with γ -globulin. Closer inspection of Figure 2 indicates that the effect is variable as indicated by a high standard deviation and a wide range of values obtained: the frequency diagram shows a gaussian distribution. After 24 hours the relative values are lower than after one hour. According to the data presented, rechromatography of fragment I on CM cellulose did not diminish its activity.

The effect of γ -globulin and fragment I prepared from anti-Salmonella paratyphi B antiserum - As the data shown in Table III and Figure 3 indicate, the activity of these preparations in promoting removal of Salmonella typhi murium from the circulation of the mouse was almost identical with that found with the anti-Salmonella typhi murium antiserum. An attempt was made to compare the effectiveness of decreasing amounts of γ -globulin and fragment I. The minimal effective amount of γ -globulin was found to be approximately $0.05 \mu\text{g}$ per mouse;

whereas that of fragment I was about 2.5 μ g per mouse, although it is evident from the frequency diagram as well as from the standard deviation, that the results with these two dosages of γ -globulin and fragment I respectively are not entirely comparable. Nevertheless, it can be said that fragment I is about 50 times less effective than γ -globulin. It is interesting to note that even the smallest concentration of γ -globulin did not show a frequency distribution similar to that obtained when testing fragment I. This suggests that the activity of fragment I is not based on the presence of some residual γ -globulin. It is noteworthy that neither γ -globulin nor fragment I derived from normal rabbit serum had any effect on the clearance of *Salmonella typhi* murium.

DISCUSSION

This results presented above clearly indicate that γ -globulin and fragment I prepared from anti-*Salmonella typhi* murium and anti-*Salmonella paratyphi* B rabbit anti-serum have an opsonizing effect on the removal of *Salmonella typhi* murium from the circulation in mice. It seems unlikely that the activity of fragment I represents contamination with whole γ -globulin for several reasons. Firstly, the preparation of fragment I proved to be uniform in the tests performed. Thus, the ultracentrifugal pattern showed a single, well defined peak with a sedimentation constant of 3.5S; and in Ouchterlony plates no indication of cross-reactions between fragment I and γ -globulin or fragment III was detected. Secondly, rechromatography on CM cellulose of the fragment I did in no way alter its physico-chemical characteristics or diminish its opsonizing activity. Thirdly, as will be discussed below, γ -globulin and fragment I exhibited a different pattern of activity indicating that the effect of fragment I would be different if it were due to a small amount of residual γ -globulin.

However, there are some differences in the effectiveness of γ -globulin and fragment I, which deserve some attention.

1. The effect of fragment I based on the amount of protein in the solution is approximately 50 to 100 times smaller than that of the γ -globulin.
2. While effective opsonization with γ -globulin seems to result in rapid removal of all organisms from the circulation within one hour, mice respond irregularly to the immunization with fragment I. Thus, some mice clear the bacteria as rapidly as those injected with γ -globulin whereas others behave like untreated controls. This variation in response is expressed by the pattern of frequency diagrams (Fig. 2 and 3) as well as by the large standard deviation (Tables II and III). There are several reasons why fragment I may possibly have a different opsonizing effect than γ -globulin. First of all, complexes of antigen with fragment I are not supposed to fix complement (29). The lack of this added protein may result in insufficient and variable opsonization, although some opsonization obviously takes place. According to Ward and Enders (34) the presence of complement accelerates phagocytosis induced by specific antibody when studied in a system containing polymorphonuclear leukocytes and encapsulated pneumococci.

Since in vitro studies were not done, no conclusion can be reached in this regard. Secondly, the procedure of obtaining fragment I may cause increased heterogeneity of the fragments with loss of binding capacity. Thirdly, since the animals were passively immunized two hours prior to the challenge infection, a difference in distribution of fragment I compared with γ -globulin could possibly account for the variance. Fourthly, it is possible that the fragment promotes only one of the steps resulting in phagocytosis. A few preliminary observations made in vitro support such an interpretation. Peritoneal macrophages from normal mice were settled on coverslips and cultured in balanced salt solution containing 10 per cent heated normal calf serum overnight. The medium was decanted and replaced by a suspension of *Salmonella typhi* murium with no addition, 2.5 $\mu\text{g/ml}$ γ -globulin or 25 mg/ml fragment I from the anti-*Salmonella paratyphi* B antiserum. Slides were incubated for one hour, fixed and stained with giemsa. While in the presence of γ -globulin most cells contained numerous bacteria with few extracellular organisms present and little or no phagocytosis took place in the absence of protein, a different picture was observed on slides which had been incubated with fragment I. Few bacilli were seen intracellularly but a large number of bacteria were aggregated in small stacks on the surface of the cells as if adhering to each other and the cell membrane. These observations suggest that fragment I can possibly enhance or facilitate the physical rapprochement of bacteria with cells, whereas the actual intake depends on the presence of the entire globulin, which in addition to fragments I and II contains fragment III. The latter may possibly influence membrane activity leading to invagination and particle intake. The latter phase may well be enhanced by complement which for its fixation on the antigen-antibody complex requires fragment III. The role of fragment III in fixation of antibody to tissues, or the lack of fixation by fragments I and II is well established (35).

SUMMARY

1. γ -globulin and fragment I were prepared from serum of rabbits which had been hyperimmunized with *Salmonella typhi* murium and *Salmonella paratyphi* B.
2. Ultracentrifugation and agar gel precipitation tests indicated that fragment I was a pure preparation.
3. The opsonizing effect of these preparations was tested by the intravenous injection into mice followed by intravenous infection with *Salmonella typhi* murium. Removal of organisms within one hour was measured by determining the remaining organisms in the circulation.
4. While no organisms were removed from the blood within one hour in control animals, γ -globulin caused complete clearance during that period of time. The effect of fragment I was intermediate between the two.
5. The difference of activity between the two preparations and this possible mode was discussed.

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TABLE I

Number of organisms circulating in the blood of normal and passively immunized mice immediately after intravenous injection of a standard inoculum of *Salmonella typhi murium*

Mouse no.	Number of organisms (log 10) per ml blood	
	controls	immunized ¹⁾
1	4.5465	4.7634
2	4.9170	4.9042
3	4.8299	4.9096
4	4.6395	4.8325
5	4.8325	4.8401
mean	4.75	4.85
s	0.15	0.05

- ¹⁾ These mice were injected intravenously with 25 μ g anti-*Salmonella typhi murium* γ -globulin two hours prior to infection.

TABLE II

Effect of anti-Salmonella typhi murium fragment I and
 γ -globulin on the clearance of Salmonella typhi murium in mice
 after 60 min and 24 hrs

Experimental group	dose in μ g	number of mice	mean nr. bact./ml (log 10)	stand. deviation
control 0 time	-	7	4.76	0.17
60 min				
control	-	17	4.26	0.57
γ -globulin	25	10	0.00	0.00
fragment I	25	16	2.20	1.09
fragment I (rechromatographed)	25	8	0.92	1.00
24 hrs				
control	-	7	1.14	0.52
γ -globulin	25	7	0.00	0.00
fragment I	25	7	0.19	0.74

TABLE III

Effect of anti-Salmonella paratyphi B fragment I and γ -globulin on the clearance of Salmonella typhi murium in mice after 60 min

Experimental group	dose in µg	number of mice	mean nr. bact./ml (log 10)	stand. deviation	
control	0 time	-	6	4.71	0.17
	60 time				
control	-	14	4.00	0.32	
γ -globulin (non-specific)	2.5	4	3.81	0.25	
γ -globulin (immune)	0.025	5	3.86	0.30	
	0.05	8	2.16	0.43	
	0.25	7	0.67	0.75	
	2.5	6	0.00	0.00	
fragment I (non-specific)	25	5	3.28	0.20	
fragment I (immune)	2.5	17	2.61	1.36	
	25	9	1.60	1.17	

FIGURE I

Agar gel precipitation (Ouchterlony) of fragments I and III with goat antisera against rabbit γ -globulin (anti RGG on the left) and rabbit fragment III (anti III on the right) in the center wells. F I and F III denote fragments I and III respectively. F I above and below the serum containing wells are samples to be examined, those to the right and the left are samples of known purity. (We are grateful to Dr. J. J. Cebra for providing us with the goat antisera and the purified samples of fragments I and III). Fragment III is contaminated with a small amount of fragment I.

a. Fragment I prepared from anti-Salmonella typhi murium antiserum



b. Fragment I prepared from anti-Salmonella paratyphi B antiserum



FIGURE 2

The effect of γ -globulin and fragment I prepared from anti-Salmonella typhi murium rabbit antiserum on the persistence of Salmonella typhi murium in the blood 1 and 24 hours after infection (see text under Materials and Methods).

a. Counts from mice killed after 1 hour

b. Counts from mice killed after 24 hours

The number beside of each column indicates the actual number falling in this category.

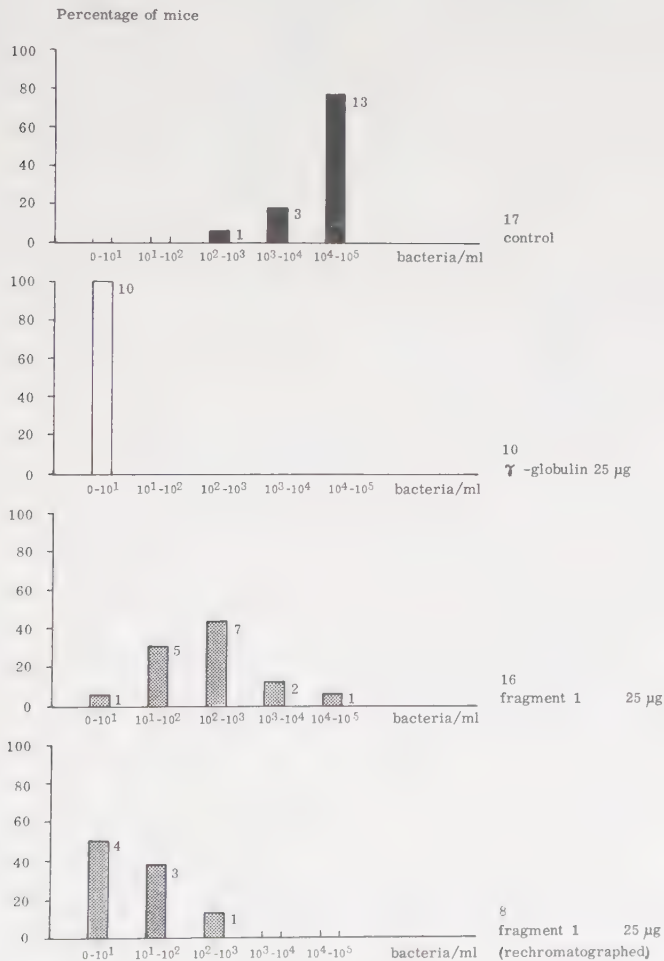


Figure 2a

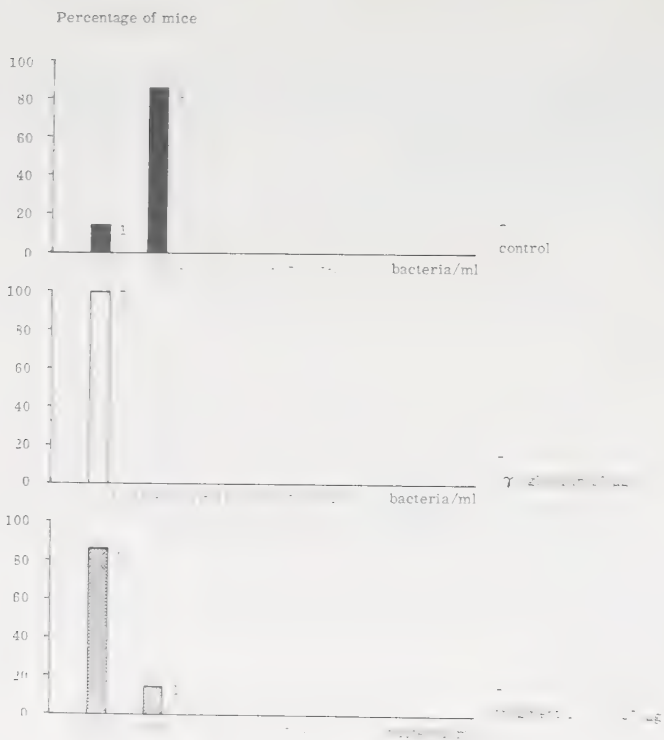
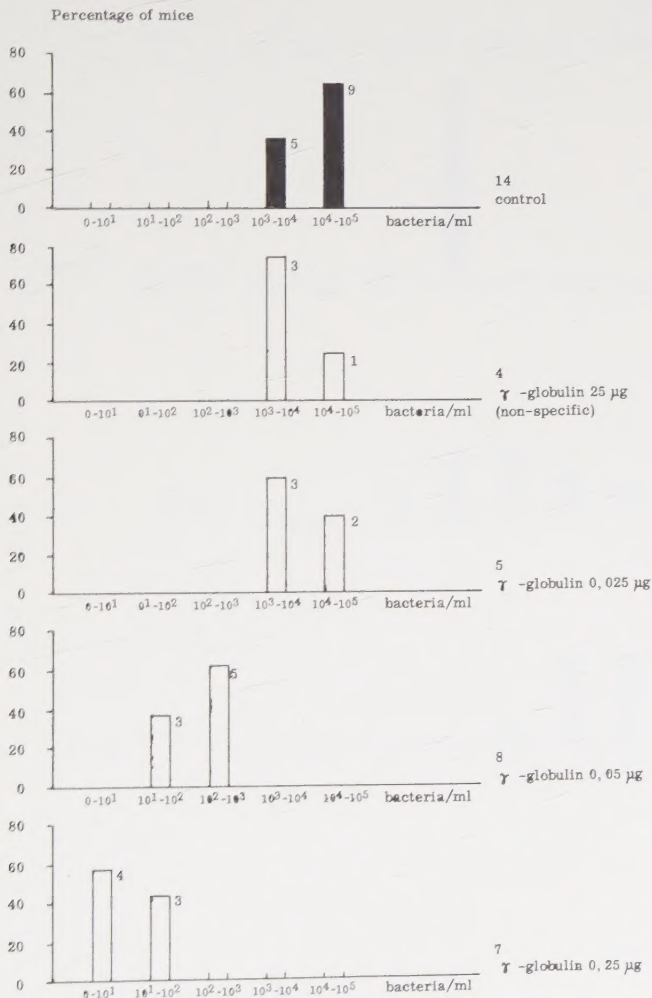


FIGURE 3

The effect of different concentrations of γ -globulin and fragment I from anti-Salmonella paratyphi B rabbit antiserum on the persistence of Salmonella typhi murium in the blood 1 hour after infection. As further controls γ -globulin and fragment I from pooled sera of normal rabbits were included in the text.



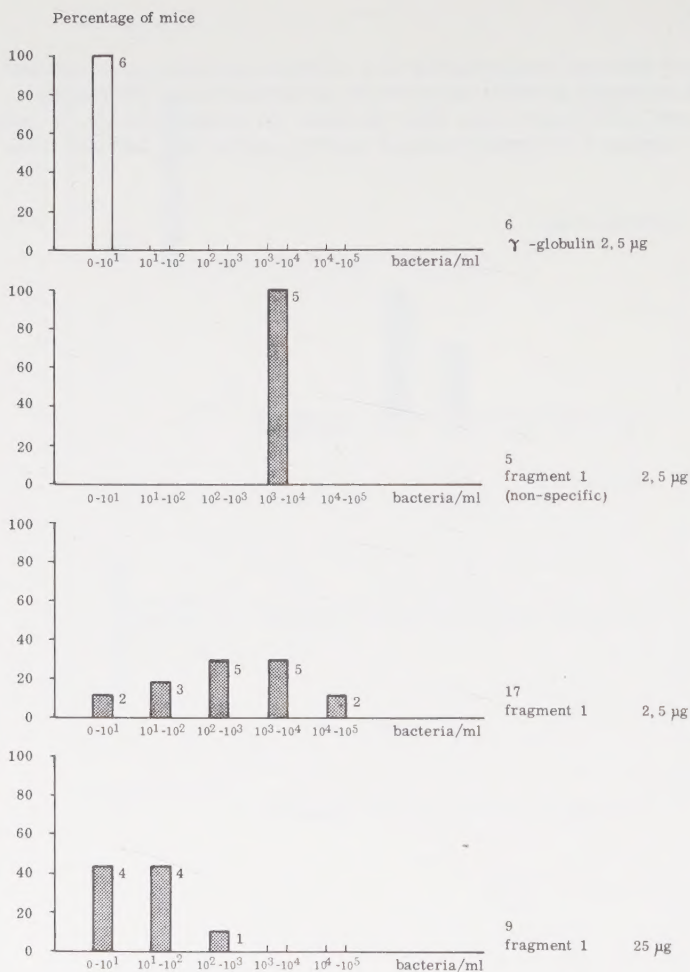


Figure 3

